SEGREGATION OF DNA IN LACTOBACILLUS ACIDOPHILUS R-26

by

NING-CHUN CHAI

B. S., Chung-Yuan Christian College

of Science and Engineering, 1963

A MASTER'S THESIS

sumbitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Genetics

Department of Physics

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1967

Approved by:

Major Professor

TABLE OF CONTENTS

INTRODUCTION....1

REVIEW OF LITERATURE....3

Histological Study of Lactobacillus Acidophilus....3

Histological Review of Autoradiography....8

Review of the Application of Fluorescent Antibody (FA)....11

MATERIAL AND METHODS....15

Bacterial Strain....15

Synthetic Medium....15

Use of Radioactive Isotope and Autoradiography....16

Transfer of Culture to Different Media....17

Bacterial Growth....17

Fluorescent Antibody Test....17

Estimation of Chromosome Number of R-26 by Means of Autoradiography ..19

Pulse labelling with ^{3}H thymine....19

Pulse prelabelling for more than two generations....19

Fluorescent Antibody (FA) and Tritiated Thymine Labelling Experiments.19

Labelling with FA immediately after the tritiated thymine pulse labelling....19

Pulse prelabelling with tritiated thymine one and a half generations before the FA labelling....20

RESULTS AND DISCUSSION....21

Presence of Segregation Conserved DNA Units....21

Association of Conserved DNA Units with Conserved Cell Surface Units..24

SUMMARY....38

ACKNOWLEDGMENTS....40

LITERATURE CITED....41

INTRODUCTION

Autoradiographic measurements of the distribution of conserved units of DNA among the progeny of exponential growing Escherchia coli has led to a model of chromosome segregation in bacteria (Lark and Bird, 1965; Lark, 1966). According to this model, when one of the strand of the DNA double helix is used as templete in replication for the first time, it becomes permanently fixed to a segregation apparatus of the cell. Thus each chromosome which has completed replication is attached to the cell by only one strand of the double helix. The newly synthesized strand is not attached.

To confirm and extend this (Lark, 1966) segregation model to Lactobacillus acidophilus R-26, R-26 cells were first labelled with radioactive thymine and then were allowed to divide in non-radioactive medium. Samples were taken at successive generations and autoradiographs were prepared. The percentage of the labelled cells were scored and the number of the conserved DNA units was estimated according to Lark and Bird (1965). Fluorescent antibodies against R-26 cells were used as a cell surface marker to follow the distribution of conserved cell surface units among the daughter cells in relation to the DNA synthesis of the cell.

Autoradiographic techniques were used in which tritiated thymine marked the radioactive DNA synthesis of the cell. These techniques have been described in detail by Caro, Van Tubergen and Forro (1958), Lark (1967). To study the permanent association of the DNA and conserved cell surface unit, cells were labelled with fluorescent antibody (FA) together with pulse labelled DNA or pulse prelabelled DNA. In the former, the templete DNA was unlabelled, while in the latter, after the pulse labelling cells were

allowed to grow for one and a half generations before the fluorescent antibody was added. Thus, half of the template DNA should be radioactive. Subsequently by observing microcolonies derived from individual cells, it was possible to measure the association of pulse label (with ^{3}H thymine) and FA label to determine to what degree labelled DNA and cells surfaces remained in association through the following cell division.

REVIEW OF LITERATURE

Histological Study of Lactobacillus Acidophilus

Lactobacillus acidophilus is a typical homofermentative lactobacillus of interest to the diary microbiologist. Orla-Jensen designated the rod-shaped lactic acid bacteria with optium growth temperatures around 40°C as Thermobacterium. Considerable amounts of acid are produced by its fermentation of carbohydrate.

Guirard, Snell and Williams (1946) investigated the nutritional role of acetate for Lactobacillus acidophilus and some other lactic acid bacteria. They found a marked effect of sodium acetate in stimulating early growth of these bacteria. This effect was not shown by other buffers and demonstrated a function for sodium acetate in bacterio-logical media other than that of a buffer.

If 1949, Hoff-Jørgensen investigated the difference in growth-promoting effect of deoxyribosides and vitamin B12 on Lactobacillus acidophilus. It was found, that for Lactobacillus audophilus R-26, (which was still named Thermobacterium acidophilus in his publication), the requirement for thymidine could not be replaced by either a B12 concentrate or a commercial liver preparation. Guanine deoxyriboside was found to have the same effect on the strain as thymidine. Later (1949) he also claimed that on a molar basis the stimulating effect of the synthetic deoxyriboside was approximately equal to that of thymidine or guanine deoxyriboside.

Actually until 1952, this organism had not been used to study DNA synthesis. Hoff-Jørgensen (1952) published a microbiological assay of deoxyribosides and deoxyribonucleic acid. In this paper, the essential requirement of Lactobacillus audophilus R-26 for a deoxyriboside was

elucidated by the finding that the amount of DNA found in the cells was equal to the amount of deoxyribosides taken up from the medium. Thus a microbiological method for the determination of deoxyribosides and DNA had been devised using this specific strain of bacteria. The method was very specific and allowed the determination of a few micrograms of DNA with a standard deviation of about 10 percent even at low concentration. The same year, Snell, Kitay and Macnutt (1952) investigated more of the lactic acid bacteria including Lactobacillus acidophilus R-26. They found that thymine deoxyriboside was an essential growth factor. Macnutt (1952) used this organism for enzymatic study, i.e. the enzymatically catalyzed transfer of the deoxyribosyl group from one purine or pyrimidine to another. He affirmed that enzyme preparations from Lactobacillus acidophilus R-26 catalized such a transfer. An interesting paper was published by Jenner and Jenner (1952). Reporting cytological study of this organism cultured in the absence of deoxyribosides or uracil. They found the ratio between the cytoplasmic and nucleoid volume was considerably increased in these cells when the growth rate was decreased by the lack of deoxyribosides in the medium. It remained constant when the limitation of growth was obtained by supressing uracil, presumably indispensable for RNA synthesis. The phenomena of elongation of the cells after being cultured in medium lacking deoxyribosides was reported in detail in this paper.

After 1952, many investigators applied Hoff-Jørgensen's microbiological assay using Lactobacillus acidophilus R-26 in their studies. Schneider (1955) used this strain as a biological assay for deoxyribosides in animals. The deoxyriboside content of the tissue extracts was estimated microbiologically with this organism. He also confirmed most of Hoff-Jørgensen's tests. In

addition, he studied the pure deoxyribosides and found them to be as active as the nucleosides in promoting the growth of this organism.

Skeggs, Wright, Gresson, MacRal, Hoffman, Wolf and Folkers (1956) presented evidence for the existance of a new factor required in the absence of acetate for the growth of a number of lactic acid bacteria including Lactobacillus acidophilus R-26. Until then, no specific study of the organism itself had been tried except the one of Jenner and Jenner (1952).

In the year 1957, a chemically defined medium had been worked out for this particular organism by Løvtrup and Roos (1957). Later, Siedler, Nayder and Schwegert (1957) made studies to improve the medium for Lactobacillus acidophilus R-26 in the assay for DNA. By this time more workers had been stimulated to study this organism.

Additional application was made to the study of animal tissue by Schneider and Rotherham (1958). Meanwhile, a group of investigators in Japan started to work on this specific organism. A series of papers were published by Okazaki and Okazak (1958a, 1958b). They studied the biological and chemical nature of the intra-cellular acid-soluble deoxyribosidic compounds and the interrelations between nucleic acid and protein synthesis in Lactobacillus audophilus R-26. It was found that the majority of acid-soluble deoxyribosidic compounds in the cells were not nucleosides, and would reveal a growth-supporting activity only after digestion with crude snake venom. The contents of the deoxyribosidic compounds were higher in the growing cells than in the resting cells. A decrease in the level of the intracellar pool of the deoxyribosidic compounds was observed when cells were transferred to a medium with a

limiting amount of deoxyriboside and allowed to synthesize DNA. It was suggested, after a study of some properties of the deoxyribosidic compounds upon chromatographic fractionation, that the main deoxyribosidic compound in the acid-soluble extract of the cells was a derivative of one of the thymidine nucleotides. The other compound was a uracil ribonucleotide. Experiments also were carried out on the effects of depletion of either deoxyribosides, uracil, or amino acids on the synthesis of DNA, RNA and protein and on the content of acid soluble compounds.

Okazaki and Okazaki (1959) continued to study the DNA synthesis in relation to RNA and protein synthesis and conluded the following: (1) Depletion of deoxyriboside inhibited DNA synthesis and cell division almost completely, but had little effect on the synthesis of RNA and protein. (2) Removing uracil from the medium caused a parallel inhibition of RNA and protein formation; but DNA synthesis was stimulated. (3) Addition of excess thymidine to cells previously starved of both deoxyriboside and uracil caused a remarkable accumulation of acid-soluble deoxyriboside compounds and an active synthesis of DNA with little or no concurrent increase of protein, RNA or cell number. Simultaneously adding chloro-phenicol and thymidine abolished the small increase in protein under such conditions with little effect on DNA. (4) Omission of amino acids from the uracil-deficient, as well as complete medium, strongly inhibited the increase of DNA.

In 1960, Sugin, Sugino, Okazaki and Okazaki made some studies on deoxyribosidic compounds, and Lactobacillus acidophilus R-26 was used as a test organism. The method was essentially the same as that described by Hoff-Jørgensen (1952) except for a few modifications. Løvtrup, Søren

and Shugar (1960) investigated the utilization of pyrimidines and pyrimidine deoxynucleosides by this organism, and claimed that either thymine or uracil could satisfy the pyrimidine requirement of the bacteria and proposed a scheme for the conversion of thymine to uracil nucleosides or nucleotides.

Biswas and Broquist (1962), using paper chromatographic examination, concluded that thymine could not be substituted for uracil in growth. Løvtrup's report (1962) was explained in terms of a possible uracil contamination. Schneider (1962) made further studies on the assay of deoxyribosyl compounds in tissue extracts and the growth of these deoxyriboside requiring bacteria was again studied. Thorne and Kodicik (1962) investigated the metabolism of acetate and movalonic acid by this organism. The requirement for acetate and its replacement by mevalonic acid was studied and (^{14}C) acetate and (^{14}C) mevalonic acid incorporation into the bacteria lipid was investigated at the same time.

A regulatory mechanism in DNA metabolism of Lactobacillus acidophilus R-26 was published by Siedler and Holtz (1963). The high specific enzyme activity formed in this organism had been partially characterized and tentatively indentified as deoxyrytidylate by differential centrifugation at low pH, by substrate specificity. The effects of varous nucleotides and deoxyribonucleic acid on the enzymatic reaction were also described in detail in this paper.

No further investigation of this organism occured until 1966, when Soska worked out a chemically defined medium, containing no folic acid. In such a medium, thymine was required in addition to deoxyriboside, purines, pyrimidines and most amino acids. A complete and careful investigation was made on the influence of the components of this medium on the growth of the

cells. A further study on the regulation of nucleic acid synthesis in this particular strain of bacteria was published by Soska and Lark (1966). Both DNA and RNA synthesis under different conditions were measured and described in detail.

In all of the foregoing studies, no one utilized autoradiographic or fluorescent antibody techniques with this microorganism. The chromosome number of Lactobacillus acidophilus R-26 can be estimated with autoradiography, by examining the population of radioactive cells in successive generations after labelling with radioactive thymine. Details of the autoradiographic technique were described by Lark (1967). The fluorescent antibody method which was introduced by Coons et. al. (1941), employs immune serum globulin labelled with fluorescent dye to locate the corresponding antigen. In this thesis, studies of Lactobacillus acidophilus R-26 using fluorescent antibody and autoradiography are attempted.

Histological Review of Autoradiography

Autoradiography was first intentionally used to locate radioactivity in minerals. In 1904, London of the Imperial Institute of Experimental Medicine published the first paper in which autoradiography was applied to the field of biology. Curie and Balthazard (1904) were using the microscopic autoradiographic method to study the distribution of inhaled radium emanation in tissue sections of a guinea pig.

In 1922, Kotzareff reported the first autoradiogram made of radium injected into a tumor of a human. After 1924, a series of studies started which have continued to the present.

Lacassagne and coworkers (1922) in Paris reported their studies of the distribution of polonium and other elements in histological specimens. Chamie (1927) published a series of papers on the use of the photographic

emulsion to study radiocolloid in dilute ionic solutions below the solubility product. As a result of Chamic's early autoradiographic work on solutions, radiocolloid in tissue can be shown and interpreted by autoradiography.

Lomholt (1930) investigated the distribution of lead in the microstructure of the tissue of mice.

The first autoradiogram of induced radioactivity was made by Groven, Govaerts and Guében (1938). Bulliard, Goundland and Mousca (1938) made the first biological autoradiogram with induced radioactivity when they demonstrated phosphorous-32 in the adrenal gland.

Hamilton, Soley and Eichorn (1940) used radioiodine to study human thyroid tissue autoradiographically. In the 1940's, Leblond published a long series of valuable papers on the thyroid and improved the autoradiographic technique. It was through his stimulation that Belanger (1940) developed the first successful method for simultaneously observing the autoradiogram and the stained tissue section under the microscope. Simultaneously with Leblond's research, a large amount of autoradiographic work was being done by Axelrod at the University of California and by Svihla at the University of Chicago. These workers really stimulated the interest in the biological field.

In recent years, many workers have developed new techniques and worked on the chemistry and physics of the emulsions to make the techniques more reliable and quantitative. It has become one of the most useful methods for studying in situ biochemical reactions at the level of the individual cell. The technique itself is relatively simple and requires little laboratory space and equipment. A specimen containing radioactive material is covered with a photographic film for a period of time. During this exposure time,

the radioactive atoms decay. The emitted radiation strikes the film, forming a latent image. Upon development. the distribution of the radioactive material within the specimen is visible as silver grains. A great variety of material may be used as specimen, such as individual cells, tissue sections, squashes or other material. In this study, individual cells of Lactobacillus acidophilus R-26 were treated with tritiated thymine and quantitative autoradiographic methods were used to follow the distribution of labelled DNA among progeny cells. The techniques of tritium autoradiography have been successfully applied to examine the various aspects of the inheritance of labelled parental DNA in E. coli by Painter, Forro and Hughes in 1958, and Forro and Wertheimer in 1960. Most of the detailed work on bacterial autoradiography has been carried out by a group of biophysicists at Yale University, which included Caro, Forro, Van Tubergen and Setlow. They published a series of papers of which the paper of Van Tubergen and Setlow written in 1961 contains a great deal of information, not only on DNA, but on RNA, protein and cell walls.

Tritium is one of isotopes most commonly used in autoradiography; it emits a β particle of 18 Kev. maximum energy. It is the lowest energy of any known isotopes. According to Caro and Van Tubergen's paper in 1962, the sensitivity for thick layers of Ilford L-4 emulsion has been estimated to be 1.3 grains per tritium β^- particle emitted in the emulsion. Use of this isotope gives excellent resolution and sensitivity.

The requirement of thymine in the absence of folic acid of Lactobacillus acidophilus R-26, makes it suitable to follow DNA synthesis by using tritiated thymine.

Review of the Application of Fluorescent Antibody (FA)

FA method was first introduced by Coons, Creech and Jones in 1941. The technique was used in a variety of ways for the study of various aspects of antigen-antibody interaction both in vitro and in vivo. In 1950, Coons and Kaplan improved the technique to localize antigen in tissue cells. They found that when an antibody of high titer is conjugated with fluorescein isocyante, the resulting FA solution can be employed as a specific histochemical stain on tissue section. Whenever antigen-antibody precipitations form, the fluoresceein-antibody is fixed. Unrelated and indifferent fluorescent proteins can be washed away. When the specimen is examined with a fluorescence microscope, the brilliant yellow-green light from the deposited fluorescence antibody reveals the presence and location of the homologous antigen.

In 1956, Moody, Goldman and Thomason used FA method to stain bacterial smears. Antiserum was prepared in rabbit against Malleomyas pseudomallei and the globulin portion was labelled with fluorecein isocyante. They found the time required for staining was 10-15 minutes using 1:2 saline-diluted labelled globulin and the staining was effected on air-dried smears prepared from cells that were fixed in various ways or not fixed. Thomason, Cherry and Moody (1956) studied the antigenic analysis of Salmonella typhosa and found that the three classes of antigenic components in cells of this species could be stained specifically both collectively and individually with appropriate labelled sera applied to bacterial smears fixed on glass slides.

Riggs (1957) introduced an isothiocyanate derivative of fluorecein with a number of qualities that made it superior to fluoreccin isocyante. Marshall, Eveland and Smith (1958) confirmed the superiority of fluorescent isothiocyanate and modified its application. Moody, Ellis and Updyke (1958) investigated grouping streptococci with fluorescent antibody. A specific fluoresceein-

labelled antibody reagent was prepared which stained all group A streptococci strains tested and strains of no other streptococcal group. The reagent was prepared by absorbing group A FA with group C organism. The adsorption removed the cross staining reaction with group C organism, but did not affect its affinity for group A cells.

Cairns (1960) studied the initiation and progress of vaccinia virus infection in tissue culture cells. He used tritiated thymine and autoradiography to show the synthesis of DNA and fluorecein-coupled antibody to demonstrate the synthesis of virus protein.

Since 1962, many papers had been published describing microbial cell wall on surface component replication as determined by FA technique. The description varied to some extent, making it quite obvious that all microorganisms do not replicate cell wall or other surface antigens in the same manner. Cole and Hahn (1962) studied the cell wall replication in Streptococcus pyrogenes. They concluded that cell wall synthesis in actively growing cultures usually occurs simultaneously at at least two sites per coccus. Each site represented stages in successive divisions. Thus, cell wall growth in Streptococcus pyrogenes is not by diffuse intercalition with old wall, but is initiated and extends both peripherally and centripctally from the coccal equator. Fox (1962) made a study on the measurement of streptococcal antigen synthesis with fluorescent antibody. He found the amount of fluorccein conjugated antibody adsorbed and eluted from cells was a function of the amount of antigen present during the process of antigenic protein synthesis. May (1962) investigated the sites of cell wall extension. He used Schizosaccharomyus pombe and applied the FA method. In this organism it was shown microscopically that new cell wall was added at a growing point at one or both ends of the cell.

May (1963) studied the distribution of cell wall label during growth and division of Salmonella typhimicorium and indicated that during growth of this organism, the absorbed antibody, and by inference the underlying cell-wall component to which it is attached, is uniformly dispersed along the length of the cell.

Chung, Hawirko and Isaac (1964) investigated the cell wall replication in Bacillus cereus and Bacillus megaterium, They found that growth of new cell wall in B. cereus was initiated near the pole, in the old wall, additional new wall segments gradually developed to form an alternating pattern of new and old wall segments. Further growth elongated the new wall and pushed the old segments apart. Separation of daughter cells appears to involve splitting of the transverse septa laid down at or near the old wall segments; growth of new cell wall of B. megaterium was initiated either at one of the poles or at the central area of the cell length. Further elongation was followed by formation of transverse septa and separation of daughter cell incorporating either old or new wall segments. They also studied cell wall replication in E. coli and Streptococcus facealis (Chung, Hawirko and Isaac, 1964b). In E. coli, the initial step in cell division was the formation of a cross wall at the cell equator, followed by the appearance of new cell wall on either side of the cross wall. A polar type of cenidirectional cell wall growth and elongation was also observed in E. coli. It was initiated by the synthesis of a ring of new cell wall material around the polar tip. A second ring was then formed at the subpolar area, during the rapid enlargement of the first ring in a single direction. Contrary to the synthesis of cell wall at multiple sites in E. coli, S. faecalis replicated new cell wall at only one site per coccus. The new segment was initiated and enlarged at the coccal equator

and was followed by the formation of a cross wall, centripetal growth and constriction to separate the daughter cells.

Cole (1964) studied the cell wall replication in Salmonella typhosa. He found that it appeared consistent only with the concept that wall replication in the Salmonellas occured by means of diffuse intercalation of new materials among old. Goos and Summers (1964) used FA techniques to observe the morphogenesis of fungi. Observation on cells of Candida albicanus indicated that wall material of the parent cell was incorporated into the wall of daughter cells or hyphol walls when these were produced. Romano and Geason (1964) studied the pattern of sheath synthesis in Sphaeotilus natans. They found that old portions of the sheath remained discretely labelled with no diminution in intensity of fluoresceince, whereas non-fluorescent new sheath materials appeared at the ends of the filaments. They concluded that sheath synthesis did not take place by diffuse intercalation, but by linear extension of pre-existing sheath.

In 1965, Cole published an article titled "Bacterial cell-wall replication followed by immunofluorescence", which gives an excellent summary of fluorescent antibody technique and its application to cell wall replication.

It is one of the purposes of this study to investigate the relationship of DNA synthesis to the replication of the cell wall of Lactobacillus acidophilus R-26 by using the FA techniques. A microcolony method was introduced in this study to ascertain the specific cell that got the fluorescent label.

MATERIAL AND METHODS

Bacterial Strain

Lactobacillus acidophilus P-26, hereafter referred to as P-26, was obtained by Soska in 1957 from Hoff-Jørgensen and has been described as a specific deoxyriboside requiring strain, suitable for biological determination of DNA and deoxyribonucleosides. This culture has been cultivated in a synthetic medium which Soska (1966) has modified from Siedler, Mayder and Schweigert (1957) and a thymine requirment was induced by omitting folic acid from the medium. The bacteria were maintained and transferred on solid agar plates containing 1.8% agar in a solution of 3.8% Difco Lactobacillus broth. R-26 was cultured in liquid synthetic medium at 39°C for experimental use.

Synthetic Medium

One liter of medium contained: potassium acetate, 15g; dextrose, 15g; guanine HCl, 30mg; adenylic acid, 16mg; cytidylic acid, 50mg; uracil, 10mg; L-asparagine, 600mg; L-glutamic acid, 500mg; glycine, 300 mg; L-leucine, 200mg; DL-almine, 200mg; L-serine, 100mg; L-valine, 100mg; L-arginine HCl, 100mg; L-tyrosine, 100mg; L-lysine HCl, 50mg; L-threonine, 50mg; L-methionine, 50mg; L-isoleucine, 50mg; L-histidine HCl, 50mg; L-tryptophan, 50mg; L-proline, 40mg; L-phenyl-alanine, 40mg; L-systeine HCl, 200mg; riboflavin, 0.5mg; nicotinamide, 0.5 mg; spermidine phosphate $6H_2O$, 5 mg; tween 80, 1000mg; sodium citrate $2H_2O$, 220mg; KH_2PO_4, 2g; $MgSO_4$ $7H_2O$, 12mg; sodium thioglycolate, 500mg; thymine, 4mg; deoxyguanosine, 8mg; folic acid (if used) 0.1mg; 5N solution of KOH used to adjust the pH; and 2.5% KCl solution was used to dilute the culture when necessary.

A basal medium, lacking all the amino acids, thioglycolic acid, deoxyguanosine, uracil and thymine, was stored as a frozen mixture. A concentrated

mixture of all the amino acids except cysteine was also stored in a frozen state. The remaining components were stored at 4°C as 50x or 100x concentrated solutions.

The compounds of interest were omitted or replaced according to the experimental conditions. Thioglycolic acid and cysteine solutions were renewed frequently since they undergo oxidation. The pH of the basal and complete medium were always adjusted to 6.9-6.95 with KOH.

Use of Radioactive Isotope and Autoradiography

Methyl labelled thymine was obtained from the New England Nuclear Corporation. It was used to study DNA synthesis and segregation of R-26. Labelled cells were washed free of radioactive material from the media by centrifugation in water. Droplets of the washed cells in water suspension were pipetted to a fairly dry agar plate. When all the liquid had been absorbed by the agar, the area containing the cells were cut out and smeared on a precleaned microscopic slide. The dry slides with the bacteria were then treated twice with cold 5% trichloroacetic acid to remove any low molecular weight labelled compounds, rinsed three times in distilled water and air dried. Kodak NTB-2 photographic emulsion was applied to the slides by dipping the slides in pre-melted film. A 6-watt safety light, with a wratten series 2 filter, was used in the process. After the emulsion was dry, slides were kept in black plastic slide boxes which were stored in a dry, dark container. After appropriate exposure time, slides were developed in Kodak D-19 developer for 2 minutes at approximately 22°C, rinsed in water, fixed in Kodak acid fixer for 4 minutes and rinsed in running tap water for half an hour. Slides were examined in a wild M-20 phase-contrast microscope at about 400x magnification and high dry condition.

Transfer of Culture to Different Media

Cultures of R-26 were transferred into media of different composition by collecting the exponentially growing cells on a membrane filter. Cells were washed with prewarmed fresh medium into which they were to be transferred and then were resuspended by washing off of the filter the new medium. The time required for the transfer never exceeded two minutes.

Bacterial Growth

Exponentially growing R-26 cells at a density of approximately 0.5×10^8 to 10^8 cells per mililiter were used as inocula. Cells were washed, diluted and suspended in an incomplete medium and then were transferred into test tubes contianing the components in question.

An 0.1 ml amount of a culture was diluted into 25ml of Abbot saline (0.9% NaCl) and was counted in a conlter counter, model B. A 30u orifice was used with a current setting of 3, a lower threshhold of 10 and a maximum gain setting. An 0.05ml volume was counted.

Fluorescent Antibody Test

Purified antibody from rabbit serum was labelled with fluorecein isothiocyanate. The labelled antibody was incubated with the pulse labelled cells for an hour at $39^{\circ}C$ in a 2.5% KCl solution. After samples were taken, both the slides of separate cells and of microcolonies were coated with Kodak NTB-2 photographic emulsion for autoradiography. After development, slides were examined under both a phase contrast and ultra-violet microscope. Pictures were taken in both microscopes to investigate the fluorescent labels and the radioactive labels.

The antiserum was obtained as follow: An overnight culture of R-26 was concentrated to a liter of 2×10^9 per ml and was fixed in 2% formaldehyde; a dosage of 1 ml was injected into the rabbit intraperitoneally. After one week, a 0.1 ml dosage was injected intravenously every other day. The injections continued for a month, after which the rabbit was bled within a few days after the last injection. Serum was separated from the blood and the antibody was purified according to Kekwick (1939 and 1940). Antibody was precipitated by adding an equal volume of 36% Na_2SO_4 and then washed with 18% Na_2SO_4 three times. The precipitate was dissolved in 2 ml of sterile water and dialyzed against cold 10^{-3} M K_2HPO_4 buffer overnight.

The labelling of the antibody with the fluorescent dye was performed as follows: A mixture of 2 ml of the serum and 2 ml of 0.05 M sodium carbonate-bicarbonate buffer at pH 8.5 was shaken approximately for 3 minutes with 5-10 mg of Celite containing about 10% of fluorescein isothio-cyanate. The mixture was centrifuged for a few minutes. The supernatant was added to a column (2.8 x 18 cm) of 'Sephadex G 25' which was prepared in advance by pouring a slurry of 'Sephadex' into a column half filled with water, allowing the gel to settle and washing afterwards with several column volumes of 0.02 M sodium phosphate buffer of pH 6.5. The column was developed with the phosphate buffer, care being taken not to disturb the exposed gel surface. A separation of the fluorescent proteins from inert dye was observed almost immediately. The stronger yellow protein band proceeded rapidly down the column while the weak inert dye band remained very close to the point of application. The labelled protein was collected almost quantitatively in approximately 6-8 ml of effluent which represented an increase in volume of 50-100 percent. Any unspecific protein was removed by absorbing the effluent with mouse cells,

and the purified antibody was left in the supernatant after centrifugation. The antibody thus labelled and purified was ready for use in the experiment.

Estimation of Chromosome Number of R-26 by Means of Autoradiography

Pulse labelling with ^{3}H thymine: Log phase growing culture were washed and transferred into tritiated thymine medium (200μc/4μg/ml). Cells were pulse labelled for 10 minutes and washed into non-radioactive medium. A 1 ml sample immediately was taken into a test tube with 1 ml of 5% cold trichloroacetic acid solution as a o generation sample. Samples were then taken up to 7 generation by following in a coulter count the number of the cells and making appropriate dilutions each generation. Samples thus taken were centrifuged and resuspended in water, cells were spread on clean microscopic slides as described in the previous part for autoradiography.

Pulse prelabelling for more than two generations: Log phase growing cells were prelabelled for more than two generations in tritiated thymine medium (40uc/4ug/ml). Cells were then transferred to non-radioactive medium and samples were taken as in the pulse labelling experiment. Slides were prepared for autoradiography as before.

Fluorescent Antibody (FA) and Tritiated Thymine Labelling Experiments

Labelling with FA immediately after the tritiated thymine pulse labelling: Exponentially growing culture of R-26 were transferred into medium lacking thymine. The cells were pulse labelled with tritiated thymine (200μc/4μg/ml) for 20 minutes. The free radioactive material was washed off with 2.5% KCl and the cells centrifuged twice in KCl. The cells were concentrated to a liter of 2-3 x 10^{8}/ml and purified fluorescent antibody was added (8μg/ml serum protein). The mixture of cells and the fluorescent antibody was incubated

at 39°C for an hour. It was then centrifuged and excess unconjugated antibody was washed away with KCl solution. The conjugated antibody and cells were diluted to a liter of 2-4 x 10^6/ml in water and 0.05 ml was spread carefully on a metal piece, which was prefixed in a lactobacillus broth agar plate that was covered with 0.4% collodion (Kellenberger, personal communication). The agar around the metal piece was precut to avoid disturbing the microcolonies that would form after incubation. Plates with the conjugated bacteria and fluorescent antibody were incubated at 39°C. Samples were taken at intervals and fixed with formaldehyde vapor for 10 minutes. Then the collodion membrane with the microcolonies was floated on a clean water surface and was picked up on a clean microscopic slide for autoradiography.

Pulse prelabelling with tritiated thymine one and a half generations before the FA labelling: This experiment was done in the same manner as the previous one, except cells were allowed to grow for one and a half generations between removal of the radioactive thymine and the fluorescent antibody labelling.

RESULTS AND DISCUSSION

Presence of Segregating Conserved DNA Units

Two experiments were carried out to estimate the number of chromosome units in each Lactobacillus acidophilus R-26 cell. A pulse labelling in the presence of 200μc/4μg/ml ^{3}H-thymine for 10 minutes and a prelabelling of 2 to 3 generations in 40μc/4μg/ml of ^{3}H-thymine. Autoradiographs were prepared, and after slides were stored in dark for sixty days, slides were developed (2 minutes in D-19 developer and fixed in Kodak fixer for 4 minutes). The results of these two experiments are shown in Table 1.

From the data in Table 1, an estimation of the chromosome number was made. The logarithm of the fraction of labelled cells were graphed against the number of subsequent generations growth in non-radioactive medium for pulse-labelled and prelabelled cells in Fig. 1.

In the case of the pulse-labelled cells, there were essentially very few unlabelled cells until after the second generation; in prelabelled cells, unlabelled cells began to appear in large amount after the third generation. In later generations, the fraction of unlabelled cells decreased exponentially in both cases as shown in Fig. 1. Extrapolation back to zero generations gave a value of about 4 conserved units in pulse-labelled cells while a value of about 8 conserved units in prelabelled cells. It is known that in pulse-labelled cells only half of the conserved units of the chromosome would be labelled, so the unlabelled cells would appear one generation earlier than in the pre-labelled culture. The data in Table 1 shows that the proportion of the labelled cells began to drop as the constant number of labelled DNA units was distributed within the increasing cell population. In the case of four conserved units of labelled DNA, all cells should be still

Table 1

Distribution of radioactive DNA in daughter cells during successive generations of growth in non-radioactive medium.

	Pulse labelled			Pre-labelled		
generations after labelling	No. cells examined	% with no grain	av. no of grains per labelled cell	No. cells examined	% with no grain	av. no. of grains per labelled cell
0	1000	1	--	1000	1	--
1	1000	1	--	1000	1	--
2	536	4.8	5.33	1000	1	--
3	574	23	2.8	562	3.02	6.5
4	539	53	2.44	516	17.6	3.2
5	540	70	1.84	515	52	2.81
6	533	81.2	1.86	505	74	2.62
7	582	89.4	1.9	504	87.3	2.5
8	535	94.4	1.9	---	---	---

Cells were pulse labelled or pre-labelled with ^{3}H-thymine as described in the text. They were then transferred to non-radioactive medium and cell division followed in the Coulter counter. Samples were taken after each doubling of the cell number. The culture density was maintained below 10^{6} cells/ml by appropriate dilution with fresh medium. Autoradiography were prepared from each sample and developed after 60 days exposure.

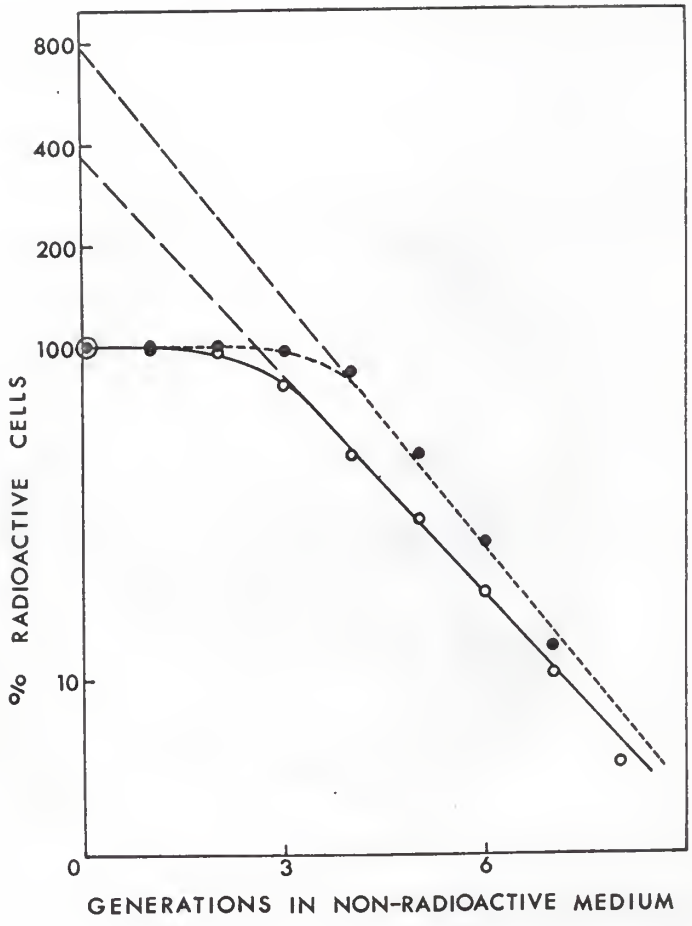

Fig. 1 The distribution of radioactive DNA among progeny cells during growth in non-radioactive medium.

The data from the experiment described in Table 1 are shown. The solid symbols on the dotted curve present data from cells prelabelled with ^{3}H thymine for three generations. The open symbols on the solid curve present data from cells pulse labelled with ^{3}H thymine.

The logarithum of the percent of labelled cells is graphed as a fraction of the number of generations of growth in non-radioactive medium. An extrapolation to zero generations yields an estimate of the percent of conserved units present. Since 100% of the population was labelled, the values of 400% or 800% indicate an average of four or eight radioactive units per cell (15).

labelled in the second generation. In the third generation unlabelled cells should appear and increase in frequency during the successive generations. In the case of eight conserved units, the drop in the labelled cells first appeared at the fourth generation. Thus with four conserved units, the unlabelled cells appeared after the cell population had increased eight fold after the incorporation of radioactivity ceased, whereas with eight conserved units a sixteen fold increase in cell number was needed. It was already known that chromosome fragmentation occured during replication in E. coli (Forro, 1965; Lark and Bird, 1965). If this is true in Lactobacillus the number of cells which have no grains would be reduced so that the labelled cells in both cases would be somewhat higher than expected.

As cells divided in non-radioactive medium, the value of the average number of grains per labelled cell decreased by a factor of two per generation until there is only one or less radioactive unit per cell.

Based on the data from these autoradiographs, it is concluded that the exponentially growing Lactobacillus acidophilus R-26 cell contains eight large conserved DNA structure or two replicating chromosomes as shown in Fig. 2.

Association of Conserved DNA Units with Conserved Cell Surface Units

R-26 cells were labelled with fluorescent antibody (FA) in two experiments. In the first one, cells were labelled with FA for one hour immediately after a 20 minutes pulse labelling of tritiated thymine. In the second experiment the labelling with FA was carried out about one and a half generations after the pulse labelling FA labelled cells were spread on collodion-covered lactobacillus broth plates and microcolonies prepared as described in Material and Method; autoradiographs were then prepared. Photographs of the same

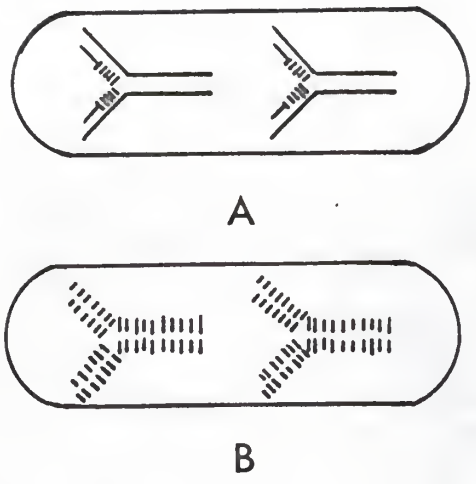

Fig. 2 A cell containing two replicating chromosomes.

(A) A pulse of radioactivity (vertical lines) labels four of the eight available conserved units.

(B) Prolonged growth in ^{3}H thymine labels all of the eight subunits of the two replicating chromosomes.

colonies were taken both with the fluorescent and phase contrast microscopes. The pictures and results of these two experiments are shown in Fig. 3 and Tables 2 and 3.

According to the data in Tables 2 and 3, only about 13% of the fluorescent labelled spots were associated with the grain clusters when fluorescent antibody was added immediately after the radioactive pulse labelling. In the experiment when R-26 cells were labelled with FA one and a half generations after the ^{3}H thymine pulse labelling, about 64% of the fluorescent spots were associated with the grain clusters.

According to the model of chromosome replication , if each chromosome is assumed to be fixed to the cell wall by one of its two component strands, this attached strand must be one which was synthesized sometime previously and is now being used as a template. This attachment will distribute the replicated chromosomes into daughter cells. After completing replication, the progeny chromosomes separate assuming their new positions in the cell or retaining their old positions according to the attachment of the template strand. Thus, in the process of serving as a template for replication, a conserved unit of DNA becomes permanently fixed to the cell. Such a fixed single strand of DNA has been postulated by Lark (1966) to be the basic unit of chromosome segregation.

When the pulse labelling of DNA immediately preceded the fluorescent antibody treatment, the parental strands which were attached to the cell should be non-radioactive. Thus, after several generations, the fluorescent labels would stay associated with the non-radioactive parental templete strands and the labels would not be associated with grain clusters. In the DNA prelabelling case, the parental DNA which attached to the cell was radioactive since it was already grown for one and a half generations before

Table 11

Association of conserved fluorescence with grains in the microcolonies formed when P-26 cells were labelled with FA immediately after the ^{3}H thymine pulse label

Estimated cell no.	No. grain clusters per colony	Fluorescent spots: Total number	Fluorescent spots: Associated with grains	Fluorescent spots: Not associated with grains
60-80	7	4	1	3
20-25	5	2	0	2
14-16	3	2	0	2
14-16	5	2	1	1
3 0-40	4	3	1	2
16-18	7	2	0	2
4 0-50	8	2	1	1
60-80	8	4	1	3
40-50	6	3	1	2
60-80	9	4	0	4
40-60	5	3	1	2
20-30	6	3	0	3
20-30	7	4	0	4
18	2	2	0	2
40-60	7	3	1	2
12	3	2	0	2
20-30	6	2	0	2
20-30	6	2	0	2
20-25	5	4	0	4
16-18	5	2	0	2
12	2	3	1	2
20-30	5	3	0	3

Table II (Continued)

Estimated cell no.	No. grain clusters per colony	Fluorescent spots: Total number	Fluorescent spots: Associated with grains	Fluorescent spots: Not associated with grains
20-30	4	3	0	3
40-50	9	3	1	2
20-30	4	3	0	3
20-25	5	2	0	2
30-40	7	3	1	2
20-25	6	3	1	2
40-50	9	2	0	2
16-20	5	2	0	2
20-25	7	2	0	2
20-30	11	3	0	3
12-14	3	1	0	1
20-30	5	4	0	4
18-20	6	3	1	2
20-25	7	2	0	2
20-25	4	1	0	3
50-60	8	3	0	1
20-25	1	2	0	2
70-80	9	6	1	5
40-50	10	1	0	1
140-160	18	2	0	2
80-100	5	2	0	2
40-50	8	2	0	2
40-50	7	2	0	2

Table II (Continued)

Estimated cell no.	No. grain clusters per colony	Fluorescent spots		
		Total number	Associated with grains	Not associated with grains
70-80	7	4	1	3
70-80	10	3	1	2
80-90	9	3	0	3
150-180	12	5	1	4
120-140	7	4	1	3
40-50	11	2	0	2
Total 51 colonies	337	139	18	121

Table III

Association of conserved fluorescence with grains in the microcolonies formed when R-26 cells were labelled with FA one and one half generations after the ^{3}H thymine pulse label

Estimated cell no.	No. grain clusters per colony	Fluorescent spots		
		Total number	Associated with grains	Not associated with grains
18-20	4	4	3	1
14	1	2	1	1
14-16	2	2	2	0
8	2	1	1	0
18	2	3	2	1
7	1	1	1	0
60-70	5	6	3	3
20-25	2	2	1	1
4 0-60	5	4	2	2
20	2	2	1	1
5 0-60	4	4	3	1
30-40	4	3	3	0
20-25	3	2	1	1
20-25	5	3	2	1
20-25	4	2	1	1
50-60	5	3	2	1
50-60	4	3	2	1
50-60	4	4	2	2
60-80	4	3	2	1
25-30	3	3	1	2
30-40	3	3	2	1

Table III (Continued)

Estimated cell no.	No. grain clusters per colony	Fluorescent spots: Total number	Fluorescent spots: Associated with grains	Fluorescent spots: Not associated with grains
14	3	3	2	1
20-30	3	2	1	1
60-80	4	3	2	1
18	3	3	1	2
80-100	5	6	3	3
20-25	3	4	2	2
20-25	3	4	3	1
80-100	5	4	2	2
80-100	4	4	3	1
60-80	5	4	2	2
20-30	5	4	3	1
40-60	4	4	3	1
60-80	6	5	3	2
20-25	4	3	2	1
20-25	4	3	2	1
60-80	5	4	1	3
40-60	3	3	2	1
25-30	4	4	2	2
60-80	5	3	2	1
60-80	5	4	3	1
30-40	5	4	2	2
50-60	5	4	3	1
20-30	2	3	1	2
18-20	4	3	1	2

Table III (Continued)

Estimated cell no.	No. grain clusters per colony	Fluorescent spots: Total number	Fluorescent spots: Associated with grains	Fluorescent spots: Not associated with grains
80-100	6	3	2	1
120-140	6	6	4	2
20-30	3	2	2	0
16	2	2	2	0
50-60	5	4	3	1
60-70	5	3	2	1
20-30	4	3	1	2
40-50	4	3	3	0
20-30	3	3	2	1
8	2	2	2	0
30-40	4	4	3	1
40-50	5	4	3	1
20-25	4	2	2	0
30-40	5	4	2	2
40-50	4	3	3	0
40-50	4	3	2	1
30-40	3	2	2	0
Total 62 colonies	237	201	129	72

Table IV

Association of conserved fluorescence with radioactive DNA

Time of ^{3}H thymine pulse	Immediately before FA	1½ Generations before FA
No. of colonies examined	51	62
Average no. of cell per colony	45	42
Total no. of fluorescent spots	139	201
Total no. of grain clusters*	337	237
No. of fluorescent spots with silver grains	18	129
Grain clusters/colony	6.6	3.8
Fluorescent spots/colony	2.7	3.2
% Fluorescent spots with grains**	13%	64%

* Grains clusters are areas in which three to five silver grains appear as a group over one bacterium.

** Association of a single silver grain with a fluorescent area was scored as positive i.e.--a fluorescent spot with silver grains.

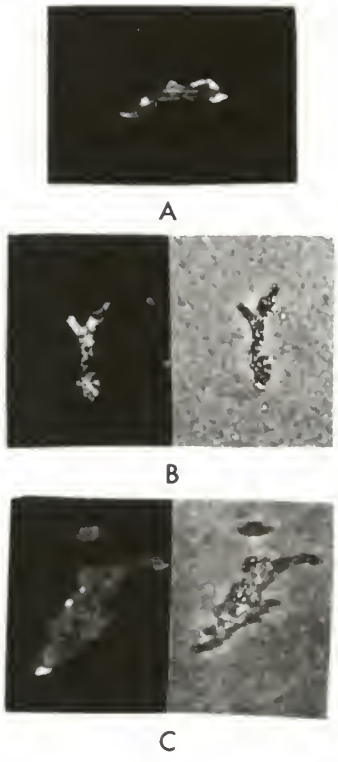

Fig. 3 Photographs of microcolonies derived from fluorescent antibody labelled cells.

(A) A photograph in the fluorescent microscope of a microcolony derived from an FA labelled cell (or cells) which was not overlaid with photographic film.

(B) A microcolony derived from a cell labelled with ^{3}H thymine and then immediately labelled with FA. This preparation is an autoradiograph. The fluorescence is shown in the photograph on the left, the silver grains appear as white dots in the phase contrast picture to the right. Note that neither of the two fluorescent spots (located in the two arms of the Y) are associated with silver grains which otherwise are distributed over the rest of the microcolony.

(C) A microcolony derived from a cell labelled with ^{3}H thymine, grown for 1½ generations in non-radioactive medium and then labelled with FA. Of the four fluorescent spots in the left hand picture, three are associated with silver grains.

labelling with fluorescent antibody and after several generations the fluorescent labels of the cell wall would remain associated with the radioactive parental DNA; that is, the fluorescence should remain associated with the grain clusters.

Thus the experimental data confirm the model. The exceptions might be caused by DNA fragmentation or failure of the original labelled cells to divide.

Theoretically, in a colony which was originally derived from one single pulse labelled R-26 cell, there should be four grain clusters per colony if the colony originated from a cell which did not divide between the time when it was labelled and when the cell was placed on solid medium. It has already been shown that there are 4 conserved radioactive units in a pulse labelled cell. (See Table 1 and Fig. 1). In the pulse prelabelled cell, because the labelling with fluorescent antibody was done one and a half generations after the pulse, the number of grain clusters should have been reduced to about 2.

Although the grain cluster in Table 4 are in relative agreement, the absolute values indicated 6-7 conserved radioactive units per pulse labelled cell rather than 4 as predicted. However, fragmentation probably is responsible for this increase. Because cells are often clumped after FA treatment, it is also impossible to be sure that each colony arises from a single cell. Therefore, the number of conserved units of cell surface per cell (fluorescent spots per cell) cannot be estimated with certainty. Nevertheless the number is probably not less than 2 or more than 4 per cell.

The value of 64% of DNA labelled fluorescent spots for pulse prelabelled cells is in good agreement with the 50% expected if original template DNA is

attached to the cell surface, since half of the templates are not radioactive and half are one generation following a pulse label (see Fig. 2A). The 13% labelled spots in the pulse labelling immediately before fluorescent antibody treatment may represent sister chromotid exchange or initiation of a new round of replication during the 20 minutes labelling period.

In general, however, we can conclude that DNA replication follows the model proposed (Fig. 4): A polynucleotide strand is attached to the cell surface, when it is first used as a template in replication.

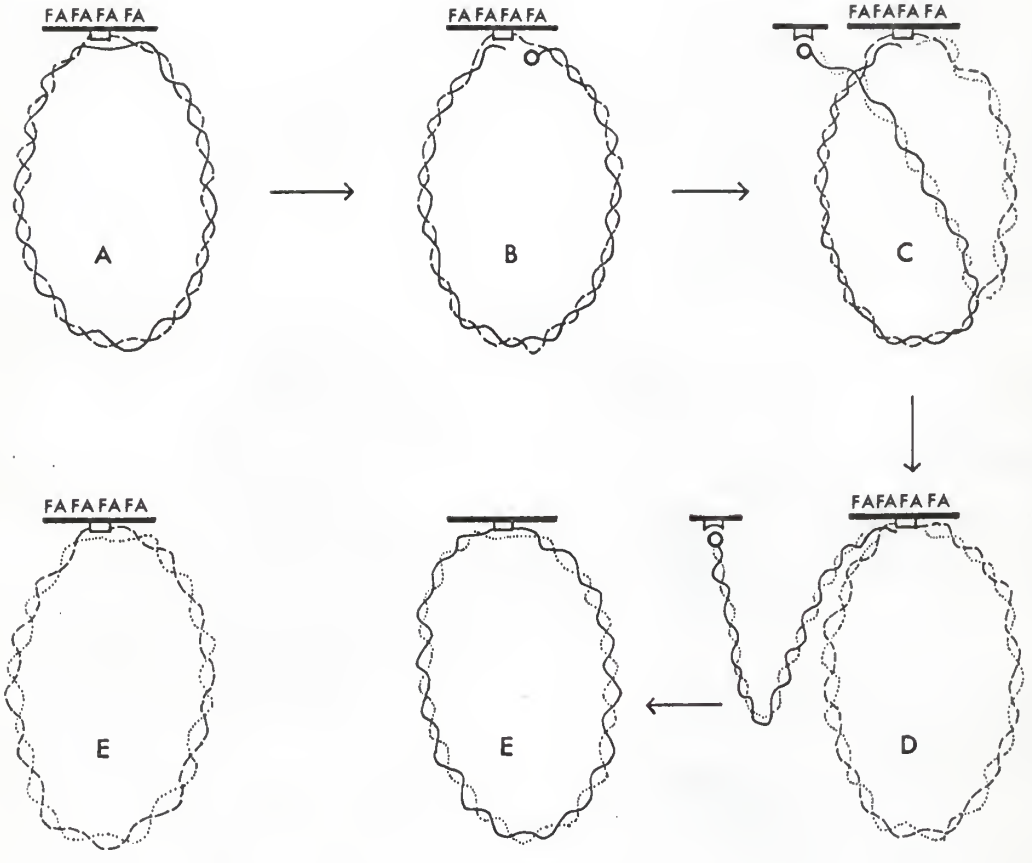

Fig. 4 A model for chromosome replication and segregation.

(A) The completed chromosome is attached to the cell surface by a single polynucleotide template strand represented by the dashed line. The cell surface is labelled with fluorescent antibody (FA).

(B-D) Replication is initiated when the non-attached recently synthesized strand (solid line) is attached to a newly synthesized portion of the cell surface (not labelled with FA).

(E) When replication is completed, the chromosome containg the original template strand (dashed line) is still attached to the FA labelled cell surface, whereas the chromosome containing the template used for the first time (solid line) is now attached to a new cell surface which is not labelled with FA.

SUMMARY

The presence of conserved units of DNA in the gram positive organism Lactobacillus acidophilus R-26 cell was determined by means of autoradiography. In a pulse labelling experiment, R-26 cells were labelled with tritiated thymine for about one fifth of its generation time. Cells were then allowed to grow in non-radioactive medium, samples were taken at successive generations and autoradiographies prepared. It was found that there were four radioactive conserved units in such pulse labelled cells.

In another experiment, R-26 cells were prelabelled with tritiated thymine for more than two generations before they were transferred into non-radioactive medium. Samples were taken at successive generations and autoradiography prepared. Eight conserved units of DNA were found which would correspond to two replicating chromosomes per cell.

Fluorescent antibody (FA) against R-26 cells were used in this study as a cell surface marker to demonstrate that portions of the cell surface were conserved during growth and cell division. The relationship between DNA and the cell surface was observed by combining autoradiographic technique with fluorescent antibody. R-26 cells were first pulse labelled with tritiated thymine and this was then either followed immediately with FA label, or cells were allowed to grow for about one and a half generations before adding the FA label. The FA treated cells were spread on solid medium plates, which were already covered with collodion, to form microcolonies.

After autoradiographic treatment, microcolonies were examined with both a UV microscope and a phase contrast microscope and the association of the fluorescent spots and the grains was scored. The consistent results in both experiments confirmed the model propsoed by Lark (1966) which states

in the replication of chromosome in bacteria, one strand of the double helix DNA becomes permanently attached to the cell surface when it is used for the first time as a template in replication.

ACKNOWLEDGMENTS

The author wishes to express her thankfulness to Professor Lark, her major professor, for his guidance and encouragement in this study.

The author wants to extend her appreciation to Dr. Kellenberger, Dr. Eberle and Dr. C. Lark for their help and advice and Dr. Weide and Dr. Bailie, for providing the ultra-violet microscope for part of this study.

She also wishes to thank Dr. Consigli and Dr. Pittenger, members of the advisory committee for their technical help and valuable comments.

LITERATURE CITED

Biswas, C., and H. P. Broquist. 1962. Inability of thymine to replace uracil for growth of thermobacterium acidophilum (Lactobacillus acidophilus) J. Bacteriol. 84: 1124.

Bonhoeffer, F., and A. Jieren. 1963. On the growth mechanism of the bacterial chromosome. J. Mol. Biol. 7:534.

Boyd, G.A. 1955. Autoradiography in biology and medicine. Academic Press Inc., New York.

Cairns, J. 1960. The initiation of vaccinia infection. Virology. 11: 603-623.

Cairns, J. 1963. The chromosome of Escherichia coli. Cold Spr. Harb. Symp. Quant. Biol. 7: 534-540.

Cairns, J. 1963. The bacteria chromosome and its manner of replication as seen by autoradiography. J. Mol. Biol. 6:208.

Caro, L. G., R. P. Van Tubergen, and F. Forro. 1958. The localization of DNA in E. coli. J. Biophys. and Biochem. cytol. 4: 491.

Caro, L. G. 1961 Localization of macromolecules in E. coli. I. DNA and proteins. J. Biophysic and Biochem. Cytol., 9: 539.

Caro, L.G., and F. Forro Jr. 1961 Localization of macromolecules in E. coli. II. RNA and its site of synthesis. J. Biophysic and Biochem. Cytol., 9:555.

Caro, L.G. 1964. High-Resolution Autoradiography in "Methods in Cell Physiology." (D. Presscott, ed.) 1: 327. Academic Press. New York and London.

Chung, K. L., R.Z. Hawirko, and P. K. Issac. 1964a. Cell wall replication I. Cell wall growth of Bacillus cereus and Bacillus metaterium. Can. J. Microbiol. 10: 43-48.

Chung, K. L., R.Z. Hawirko, and P.K. Isaac. 1964b. Cell wall replication II. Cell wall growth and cross wall formation of Escherichia coli and streplococcus faecalis. Can. J. Microbiol. 10: 473.

Cole, R.M., and J.J. Hahn. 1962. Cell wall replication in streptococcus pyogenes. Science. 135: 722.

Cole, R. M. 1964. Cell wall replication in Salmonella typhosa. Science. 143: 820.

Cole, R. M. 1965. Bacterial cell-wall replication followed by immunofluorescence. Bact. Rev. 29: 326-344.

Coons, A. H., H. J. Creech, and R. N. Jones. 1941. Immunological properties of an antibody containing a fluorescent group. Proc. Soc. Exptl. Biol. Med. 47: 200.

Coons, A. H., H. J. Creech, R.N. Jones, and E. Berliner. 1942. The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. J. Immunol. 45: 159.

Coons, A. H., and M. H. Kaplan, 1950. Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. J. Exptl. Med. 91: 1.

Coons, A. H. 1958. Fluorescent Antibody Methods in "General Cytochemical Methods." 1: 400. Academic Press Inc. New York.

Eberle, H., and K. G. Lark. 1966. Chromosome segregation in Bacillus subtilis. J. Mol. Biol. 22: 183.

Eberle, H., and K. G. Lark. 1967. Chromosome replication in Bacillus subtilis cultures growing at different rates. Proc. Nat. Acad. Sci. U.S. 57: 95.

Forro, F. Jr., and S. A. Wertheimer. 1960. The organization and replication of deoxyribonucleic acid in thymine-deficient strain of E. coli. Biochem. Biophys. Acta. 40: 9.

Forro, F. Jr. 1965. Autoradiographic studies of bacterial chromosome replication in amino acid deficient Escherichia coli 15T. Biophysical J. 5: 629.

Hoff-Jørgensen, E. 1952. A microbiological assay of deoxyribosides and DNA. Biochem. J. 50: 450.

Jeener, H., and R. Jeener, 1952. Cytological study of thermobacterium acidophilus cultured in the absence of deoxyribosides or uracil. Exptl. cell Res. 3: 675.

Kekwick, R. A. The electronelio analysis of normal Ruman serum. 1939. Biochemical J. 33: 1122.

Kekwick, R. A. 1940. The serum protein in multiple myelomatosis. Biochem. J. 34: 1248.

Kellenberger, E. and W. Arber. 1957. Electron Microscopical studies of phage multiplication 1. A method for quantitative analysis of particle suspensions. Virology. 3: 245.

Lark. C. 1967. The measurement of bacterial DNA synthesis and segregation by means of autoradiography (in press).

Lark, K.G., and R. E. Bird. 1965. Segregation of the conserved units of DNA in Escherichia coli. Proc. Natl. Acad. Sci. U.S. 54: 1444.

Lark, K. G., and C. Lark. 1965. Regulation of chromosome replication in Escherichia coli: Alternate replication of two chromosomes at slow growth rates. J. Mol. Biol. 13: 105.

Lark, K. G. 1966. Regulation of chromosome replication and segregation in bacteria. Bact. Rev. 30: 30.

Lark, K. G., R. A. Consigli, and H. C. Minocha. 1966. Segregation of sister chromatids in mammalism cells. Science. 154: 202.

Lark, K.G., H. Eberle, R.A. Consigli, H.C. Minocha, N. Chai, and C. Lark. 1967. Chromosome segregation and the regulation of DNA replication. To be published in the book Organizational Biosynthesis Bicentennial Symposium.

Løvtrup, S., and K. Roos. 1957. A microbiological determination of DNA. The growth of thermobacterium acidophilum on a chemically defined medium. Cell Rev. Suppl. 4: 269.

Løvtrup, S., and D. Schugar. 1961. Utilization of pyrimidines and pyrimidine deoxyribonucleosides by thermobacterium acidophilum. (Lactobacillus acidophilus). J. Bacteriol. 82: 623.

Macnutt, W. S. 1952. The enzymatically catalyzed transfer of the deoxyribosyl group from one purine or pyrimidine to another. Biochem. J. 50: 384.

Marschall, J. D., W. C. Eveland, and G. W. Smith. 1958. Superiority of fluorescent isothiosyanate (Riggo) for fluorescent-antibody technic with a modification of its application. Proc. Soc. Exptl. Biol. Med. 98: 898.

May, J. W. 1962. Sites of cell-wall extension demonstrated by the use of fluorescent antibody. Exptl. Cell. res. 27: 170.

May, J. W. 1963. The distribution of cell wall label during growth and division of Salmonella typhimurium. Exptl. Cell. Res. 31: 217.

Meselson, M., and F. W. Stahl. 1958. The replication of DNA in Escherichia coli. Proc. Natl. Acad. Sci. U.S. 44: 671.

Moody, M. D., M. Goldman, and B. M. Thomason. 1956. Staining bacterial smears with fluorescent antibody. I. General methods for Malleomyces pseudomallei. J. Bacteriol. 72: 357.

Moody, M. D., E. C. Ellis, and E. L. Updyke. 1958. Staining bacterial smears with fluorescent antibody. IV. Grouping streptococci with fluorescent antibody. J. Bacteriol. 75: 553.

Nairn, R. C. 1962. Fluorescent Protein Tracing. E. & S. Livingstone LTD. Edinburgh and London.

Okazaki, R., and T. Okazaki. 1958a. Studies of DNA synthesis and cell growth in the deoxyriboside-requiring bacteria lactobacillus acidophilus. I. Biological and chemical nature of the intra-cellular acid-soluble deoxyribosidic compounds. Biochim. Biophys. Acta. 28: 470.

Okazaki, T., and R. Okazaki. 1958b. Interrelations between nucleic acid and protein synthesis in lactobacillus acidophilus. Biochim. Biophys. Acta. 29: 211.

Okazaki, T., and R. Okazaki. 1959. Studies of DNA synthesis and cell growth in the deoxyriboside requiring bacteria lactobacillus acidophilus. II. DNA synthesis in relation to RNA and protein synthesis. Biochim. Biophys. Acta. 35: 434 & 443.

Painter, R. B., F. Forro Jr., and W. L. Hughes, 1958. Distribution of tritium labelled thymidine in Escherichia coli during cell multiplication. Nature. 183: 328.

Roneano, A. H., and D. J. Geason. 1964. Pattern of sheath synthesis in sphaerolilus natans. J. Bacteriol. 88: 1145.

Schaeechter, M. M., W. Bentzon, and Maaloe. 1959. Synthesis of DNA during the division cycle of bacteria. Nature. 183: 1207.

Schneider, W. C., and J. Rotherham. 1958. Phosphorous compounds in animal tissues. J. Biol. Chem. 232: 853. 233: 948.

Schneider, W. C. 1962. Further studies on the assay of deoxyribosyl compounds in tissue extracts. J. Biol. Chem. 237: 1405.

Siedler, A. J., F. A. Nayder, and B. S. Schweigert. 1957. Studies on improvement in the medium for lactobacillus acidophilus in the assay for DNA. J. Bacteriol. 73: 670.

Siedler, A. J., and B. S. Schweigert. 1960. The effect of ribonucleotides on the deoxyribonucleic acid metabolism of lactobacillus acidophilus R-26. J. Biol. Chem. 235: 758.

Siedler, A. J., and M. T. Holtz. 1963. Regulatory mechanism in DNA metabolism of lactobacillus acidophilus R-26. J. Biol. Chem. 238: 697.

Skeggs, H. E., and L. D. Wright, E. L. Gresson, G. E. MacRal, C. H. Hoffman, D. Wolf, and K. Folkers. 1956. Discovery of a new acetate-replacing factor. J. Bacteriol. 72: 519.

Soska, J., 1966. Growth of lactobacillus acidophilus in the absence of folic acid. J. Bacteriol. 91: 1840.

Soska, J., and K. G. Lark. 1966. Regulation of nucleic acid synthesis in lactobacillus acidophilus R-26. Biochim. Biophys. Acta. 119: 526.

Sugino, Y., N. Sugino, R. Okazaki, and T. Okazaki. 1960. Studies on deoxyribosidic compounds. I. Modified microbioassay method and its application to sea urchin eggs and several other materials. Biochim. Biophys. Acta. 40: 417.

Thomason, B. M., W. B. Cherry, and M. D. Moody. 1957. Staining bacterial smears with fluorescent antibody. III. Antigenic analysis of salmonella typhosa. J. Bacterol. 74: 525.

Thorne, J. I., and E. Kodicek. 1962. The metabolism of acetate and mevalonic acid in Lactobacilli. I. The effect of acetate and mevalyonic acid on growth. II. The incorporation of (^{14}C) acetate and (^{14}C) mevalonic acid into the bacteria lipids. Biochim. Biophys. Acta. 59: 273.

Van Tubergen, R. P., and R. B. Setlow. 1961. Quantitative radioantographic studies on exponential growing cultures of Escherchia coli. Biophys. J. 6: 589.

SEGREGATION OF DNA IN *LACTOBACILLUS ACIDOPHILUS* R-26

by

MING-CHUN CHAI

B. S., Chung-Yuan Christian College of Science and Engineering, 1963

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Genetics

Department of Physics

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1967

The permanent association of DNA and the conserved cell surface units in the gram positive organism Lactobacillus acidophilus R-26 was investigated by combining the fluorescent antibody treatment with the autoradiographic technique. It was necessary to measure the segregation of DNA in Lactobacillus before beginning experiments with fluorescent antibody (FA). This was done by growing cells in medium containing radioactive thymine for three generations and then transferring them to non-radioactive medium for further growth. Samples were taken at successive generations and autoradiography were prepared. The segregation of the radioactive DNA into progeny cells were examined. It was found that such exponentially growing cells contained eight conserved units of DNA. Since there were two new polynucleotide strand being synthesized which were initiated by the two original strands of the DNA double helix, four conserved units must have been present in each replicating chromosome. Thus the eight conserved units would correspond to two replicating chromosomes per cell.

Fluorescent antibodies prepared against this organism were used to demonstrate that portions of the cell surface were conserved during growth and cell division. Cells were first labelled with a pulse of radioactive thymine and this was followed either immediately with FA label, or cells were allowed to grow for approximately one and a half generations before the FA label was added. In the former, the parental DNA strands were not radioactive, whereas in the latter, half of the parental strands were radioactive. These FA treated cells were allowed to form microcolonies on solid medium which was precovered with collodion, then the collodion membrane with the attached microcolonies were floated on water surface and picked up on microscope slides. These slides were covered with photographic emulsion and stored in a cool and dry container until development. Subsequently the same microcolonies were examined microscopically with both fluorescence and phase contrast optics to determine the

extent of association of the fluorescent spots and the grains.

The results were consistent in both experiments in demonstrating that when DNA was synthesized, it was not immediately associated with the cell surface. Instead it became associated with the cell surface synthesized during subsequent growth. In the experiment of pulse labelling followed by FA treatment, the results showed that most fluorescent spots were not associated with the grains which were markers of the radioactive DNA, that is, the old cell surface which had the FA as a marker stayed with the template DNA strand which was not radioactive. On the other hand, in the experiment in which cells were allowed to grow for one and a half generations after the pulse labelling, the results showed that more than half of the fluorescent spots were associated with the grains which corresponded to the half expected radioactive labelled DNA strands. These results are consistent with a model in which DNA becomes permanently fixed to the cell surface when it is used for the first time as a template in replication.